AD-A151 727 RAPID METHODS FOR THE LABORATORY IDENTIFICATION OF PATHOGENIC MICROORGANISMS(U) LOUISVILLE UNIV KY R J DOYLE ET AL. SEP 82 DAMD17-81-C-1028 1/1

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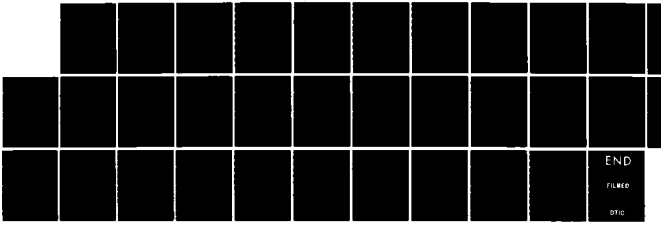

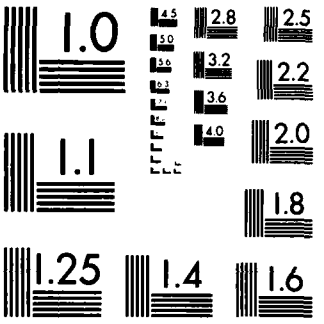

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RAPID METHODS FOR THE LABORATORY IDENTIFICATION OF PATHOGENIC MICROORGANISMS

ANNUAL REPORT

Ronald J. Doyle, Ph.D.
Kenneth F. Keller, Ph.D.

September 1982

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-81-C-1028

University of Louisville
Louisville, Kentucky 40292

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Rapid Methods for the Laboratory Identification of Pathogenic Microorganisms		5. TYPE OF REPORT & PERIOD COVERED Annual Report (1 Aug. 81-31 Aug. 82) 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ronald J. Doyle, Ph.D. Kenneth F. Keller, Ph.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD17-81-C-1028
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Louisville Louisville, Kentucky 40292		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102A.3M161102BS10.AN.058
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701-5012		12. REPORT DATE September 1982 13. NUMBER OF PAGES 37
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Lectins; Rapid Identification; Bacillus anthracis; Cryptococcus neoformans; Neisseria meningitidis.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Our studies to date have demonstrated the potential for developing a field-type kit for the rapid identification of Bacillus anthracis. We have shown that certain lectins will selectively interact with B. anthracis, but not with other species of the Bacillus genus. This finding provides the basis for devising more sensitive assays, such as an enzyme-linked lectinosorbent test. A most significant observation is the fact B. anthracis spores also exhibited a similar lectin specificity. ORIGINATOR-SUPPLIED KEYWORDS!

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SUMMARY

Many pathogens, especially those of interest to the military, often require lengthy periods of time (24 hrs-days) to identify in the laboratory. The purpose of this investigation is to determine the feasibility of employing lectins as diagnostic reagents for the rapid identification of certain pathogenic microorganisms. The rationale being that tests employing lectins as agglutinins or selective adsorbents, may be readily amenable for use in the field.

In the present research, batteries of lectins were mixed with the bacterial and/or yeast cells, incubated on a rotary shaker for periods up to 10 mins, then examined for patterns of agglutination. All parameters that are known to affect agglutination such as concentrations of reactants, time, temp, etc. were studied in order to obtain optimal reactivity. All tests were performed on organisms removed from primary isolation plates. In addition to the lectin agglutination studies, several substrates were evaluated in terms of their ability to be rapidly hydrolyzed by γ-glutamyl aminopeptidase, an enzyme unique for meningococcus among all the neisseria species. The rates of hydrolysis were determined visually by noting the earliest development of a bluish color.

Our studies to date have demonstrated the potential for developing a field-type kit for the rapid identification of Bacillus anthracis. We have shown that certain lectins will selectively interact with B. anthracis but not with other species of the Bacillus genus. This finding provides the basis for devising more sensitive assays, such as an enzyme-linked lectin-sorbent test. A most significant observation is the fact B. anthracis spores also exhibited a similar lectin specificity.

As a result of our work with Neisseria meningitidis we believe a significant breakthrough has been made in terms of developing a rapid diagnostic procedure for epidemic meningitis. The test is based on the detection of a rather unique enzyme (γ-glutamyl amino-peptidase) that is produced only by N. meningitidis and not any of the other neisseria species. We found that γ-glutamyl-4-methoxy-β-naphthalamide was a superior substrate for the detection of this enzyme. The presence of this enzyme in spinal fluid can be demonstrated in 20 mins. (blue color) after addition of the substrate. To date, 110 spinal fluids from patients have been tested and six were found to be positive; there appears to be a 100% correlation with the clinical laboratory diagnoses.

In addition to our studies on bacteria-lectin interactions, we also investigated the feasibility of using lectins for the differentiation of clinically important yeasts. Our data shows that all of the yeasts tested are agglutinable with the exception of Crytococcus neoformans. Thus the nonagglutinability of c. neoformans provides the basis for a rapid means of identification.

FOREWORD

No copyrighted material has been quoted in this report, also

No animals were used in these experiments.

TABLE OF CONTENTS

PROGRESS REPORT

Progress recorded on the following pages constitutes results obtained in the past twelve months. Earliest work will not be presented as it was described last year. In order to lucidly present the results short narratives will be offered, followed by the actual data.

The Problem and Background

The U.S. Army Medical Research Command, Ft. Detrick, MD has supported this research since February 1, 1981. The objective of the research has been to study new means for the rapid detection of microbial pathogens considered important by the U.S. Army. In the early phases of the studies, we developed technology for the use of plant proteins, called lectins, in diagnostic microbiology. We studied lectin interactions with Staphylococcus, Legionella, Streptococcus, fungi and other microorganisms. This work resulted in several publications. While this work may not have immediate application to military needs, we were able to use the technology and ideas to directly assay for an important pathogen, Bacillus anthracis. The lectin test is simply, rapid and highly specific for B. anthracis.

As this work has progressed, new ideas have emerged. It has been reported that certain bacteria and fungi have aminopeptidases. Included are Bacillus anthracis, Salmonella and Clostridium. The literature showed that each organism possessed a specific type(s) of aminopeptidase. A paper by Vedros and Hoke (1981) showed that Neisseria meningitidis possessed a gamma glutamyl amino-peptidase (γ Gap). We used γ-glutamyl-β-naphthalamide as a substrate and coupled the free β-naphthalamine to a dye in order to visually determine enzymatic activity. We reasoned that such assays could be useful for Army needs. Although naph-thalamides have been used for years for peptidase assays, they tend to be somewhat sluggish to hydrolysis. We found that 4-methoxy derivatives were readily hydrolyzed by the appropriate enzymes. Thus, when gamma-glutamyl-4-methoxy-β-naphthalamide was mixed with Neisseria meningitidis, it was found that the rate of hydrolysis greatly exceeded that of the unsubstituted γ Gap. The reason for this is simple, we think: the 4-methoxy serves to lower the energy of the gamma glutamyl peptide bond, resulting in greatly lowered activation energies. Using these results, we were able to design and test a new rapid assay for N. meningitidis in spinal fluids. We hope to similarly use 4-methoxynaphthalamides in the study of selected pathogens of military interest. Some of our preliminary results are included in the progress section. Thus, our work for the next year will emphasize the lectin-Bacillus testing, lectin interactions with other pathogens and the probing of specific enzymatic assays for selected pathogens.

Lectin-Bacillus interactions

Vegetative cells of Bacillus species were obtained by growth in a rich medium. The cells were harvested by centrifugation, washed and suspended in phosphate-saline buffer (PBS). Cells (Table 2) and lectins (Table 1) were mixed in various proportions and agglutination patterns observed (Tables 3, 4, and 5). It is emphasized that the results were obtained by use of the rapid slide assay (10 min with gentle shaking at room temperature). The important results are shown in Table 3, where it is seen that B. anthracis can be selectively agglu-tinated. Not a single other Bacillus possessed the same kinds of reactivities with lectins. Significantly, B. cereus and B. thuringiensis strains did not agglutinate with the galactose (N-acetylgalactosanine-specific lectins.) Further-more, neither B. cereus 23260 nor B. cereus 19637, both of which cross react

antigenically with B. anthracis, were capable of agglutinating with the same lectins. We view these results as very positive in providing a means to rapidly identify B. anthracis.

We must keep in mind that although many lectins may have the same "specificity", it does not mean that they all will agglutinate a particular bacterium. As an example, lectins from Maclura pomifera and Sophora japonica, both galactose-specific, would not agglutinate B. anthracis. Factors, such as stereochemistry, adjacent hydrophobic residues and receptor densities are important. These factors tend to enhance the specificity of lectins for microorganisms, rather than decrease.

One problem in the foregoing series of experiments has been the occasional autoagglutination of the organisms. When autoagglutination was too severe it was impossible to obtain clear-cut results. However, for the data shown above, autoagglutination was not considered pronounced enough to prevent reliable decisions on lectin agglutinability. We have been able to show that boiling or autoclaving largely prevents the autoagglutination (Table 6). We carry continuing studies on preventing the autoagglutinations. The data shown in Table 6 represents results from cell batches which gave severe autoagglutination and are not to be regarded as typical.

When spores were interacted with the lectins it was found that many of the spores retained lectin receptors (Table 7). Importantly, B. anthracis spores demonstrated the lectin agglutination patterns as the vegetative cells. In some cases, new lectin-reactive sites were exposed as shown by the agglutinations with HAA lectin. It is presently unknown whether the lectins are binding to the same kinds of molecules on the B. anthracis spore surface as on the vegetative cell surface. The significant observation, in terms of diagnosis, is that some lectins tend to selectively agglutinate B. anthracis cells or spores.

Additional studies on the B. anthracis cell surface were performed. These included results from chemical analyses of cell walls (Table 8), susceptibilities of the organisms to lysozyme and mutanolysin (Table 9), deregulation of the autolysins (Table 10), induction of lysozyme susceptibility by N-acetylation (Figure 1) and proof that the B. anthracis cell wall is N-unacetylated (Table 11). All of these studies were performed with the view in mind of increasing our knowledge of the cell surface of B. anthracis. Collectively, these results are highly important when considering the choices of newly discovered lectins for specific agglutinations. In addition, the results may prove significant for understanding why there is antigenic cross-reactivity between B. anthracis and certain B. cereus.

Our present view is that we now have developed the basic results needed to design a test kit for B. anthracis. A proposed simple and sensitive procedure is outlined on page 7.

Proposed assay kit for Bacillus anthracis,
based on enzyme-linked lectinosorbent assay (ELLA)

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1. Adsorb soybean lectin (SBA) on polystyrene plate. The plate may be rinsed and dried for long term storage and stability.

2. Re-hydrate plate with tap water (should take 1-2 drops water per microtiter well if this kind of plate proves useful).

3. Rinse plate 2-3 times with water, then add suspected sample of B. anthracis. Projected sample would be a suspension of cells or spores in dilute Triton X-100 or Tween - 80. Permit adsorption of cells (or spores or both) with the surface of the plate).

4. Rinse plate with several drops of tap water. Add 1-2 drops of a solution of horseradish peroxidase-conjugated SBA (this may exist as a freeze-dried sample which has been freshly re-hydrated with water). Allow time (5-10 min) for interaction, then rinse plate again with water.

5. Add 1 drop of ABTS reagent (this is a chemical that can be stored in dried form and freshly rehydrated, 2, 2'-azino-di-[3-ethyl-benythizoline sulfonate) and 1 drop 30% H_2O_2 solution.

6. If yellow color appears (actual λ max-415 mm), the sample probably contained B. anthracis.

7. Appropriate controls should be run. These are a killed suspension of B. anthracis cells (positive control) and B. subtilis (a convenient negative control).

8. Time for completion of test depends on temperature (assays should routinely be conducted at 21°C or above), time and density of cells added (or spores). Probably most routine assays could be completed and read in 60-90 min.

9. Possible modifications. We may be able to design a "dipstick" test. This would depend on the ability of cellulose filter segments to adsorb SBA or BSA-I. It may be possible to substitute SBA-alkaline phosphatse conjugates for the SBA-horseradish peroxidase conjugate. The substrate would then be the stable p-nitrophenyl phosphate. We could then circumvent the hydrogen peroxide. Other enzymes which can expediently be linked to SBA include β-galactosidase and glucose oxidase. The ultimate design is to be centered around stable reagents (preferably freeze-dried) which can be mixed and then observed for color development in a reasonable period of time.

Neisseria meningitidis studies

As described briefly in the project overview (page 5) of the proposal, we reasoned that new and specific diagnostic procedures could be developed to take advantage of the presence of aminopeptidases in certain microorganisms. We found that γ-glutamyl-4-methoxy-β-naphthalamide was a superior substrate for the γ-glutamyl aminopeptidase (γ Gap) of N. meningitidis. We worked out a procedure to test for N. meningitidis in the spinal fluids of children. Table 17 summarizes some of our results and describes the assay conditions. To date, the test has been regarded as 100% accurate, and does not suffer from interference by other microorganisms which commonly cause meningitidis (Table 18). We propose this method for the diagnosis of N. meningitidis - induced meningitis in military populations. We recognize that much work remains to be done to ensure that all factors have been considered in the assay, but presently view the method as a breakthrough.

As regards the military significance of the foregoing results when agents of BW are considered, several considerations must be raised. We embarked on this little study to test the hypothesis that 4-methoxy derivatives of β-naphthalamides could be readily hydrolyzed by the appropriate enzymes. We believe that such results may ultimately lead to highly specific reagents to rapidly distinguish between bacteria and fungi. Such assays may also be coupled to lectin assays to enhance selectivity and increase sensitivities. These promising results will be followed up in the coming year. Our plan on these studies is to critically examine the literature for reports of peptidase activities in potential agents of BW. If the literature is promising, we will obtain the appropriate 4-methoxy derivatives of β-naphthalamide and perform preliminary assays on rates of hydrolysis, etc. As indicated in the Overview, the 4-methoxy derivatives should constitute a new class of easily hydrolyzed substrates for aminopeptidases. We emphasize that this aspect of the work will not have the highest priority, but we would be at fault not to pursue such promising reagents with some effort.

Francisella tularensis and Brucella Interactions with Lectins and Plant Extracts

Objective:

The purpose of these experiments was to determine if lectins could be used for the rapid identification of Francisella tularensis as well as the three species of Brucella.

Materials and Methods:

F. tularensis whole cell antigen was initially supplied by the U.S. Army Laboratories at Ft. Detrick. The organisms had been previously killed by gamma irradiation. Other lots of tularensis antigen were purchased from commercial sources (BBL and Difco). Thethree Brucella species: suis abortus, and melitensis were also purchased from BBL and Difco. Lectins were obtained from E-Y Laboratories in San Mateo, California. Plant extracts containing lectin-like substances were prepared in our own laboratories The lectin agglutination tests were performed in Boerner slide wells and the results were read macroscopically after 5 mins on a rotary shaker.

Progress:

Some 30 lectins, and 7 plant extracts have been used in a ran[illegible] screening for agglutinating activity with Francisella tularensis and the three species of Brucella Lectins were tested at varying concentrations. Those lectins and/or extracts that showed agglutinating activity, as well as some representatives of the nonreactive agglutinins, are shown in Table 12.

When the same agglutinins that were used in the preliminary screening experiments were tested against whole cell antigens obtained from different sources, considerable variations in reactivity were observed (See Table 13). These representative data strongly suggest that the preliminary screening data were not reproducible because of antigenic variation.

Future Studies:

In order to test the hypothesis that our preliminary data were not reproducible because of differences in antigen preparations, we propose to use in future experiments organisms that are killed by antibiotic treatment rather than formalin. Lee Laboratories in Georgia has offered to prepare these antigens for us according to our specifications. After receiving the new antigens, we will repeat our earlier work using the same lectins and plant extracts. In addition, we will test some new lectins recently isolated by our laboratory.

LECTIN-YEAST INTERACTIONS

During the past year, we have continued working on the problem of differentiating the clinically important yeasts. As the following data indicates, we have developed a rapid lectin agglutination test to identify the pathogenic yeast Cryptococcus neoformans. This encapsulated yeast is the etiologic agent of Cryptococcal meningitis, and as is true for any causative agent of meningitis, its rapid identification is of utmost importance from a clinical point of view. In the event that a direct microscopic examination of a spinal fluid is negative for this yeast, then one must rely on cultural procedures to establish a definitive diagnosis. In addition to primary isolation of the organism, conventional methods require a series of subcultures and at least overnight incubation in order to read the biochemical tests results. The rapid lectin test we propose can be performed in approximately 1 1/2 hours, thus, it provides a diagnosis 12 to 24 hours sooner than conventional culture procedures.

Although our initial objective was to try to find a lectin that would selectively agglutinate Cryptococcus neoformans, none could be found. Thus, its nonagglutinability provides the basis for an identification procedure.

Test Organisms: All yeasts were fresh clinical isolates furnished by local hospital laboratories. When received in our lab, one subculture of each isolate was made on Saborauds medium prior to testing. Preparatory to performing the lectin agglutination tests, organisms were suspended in PBS (pH 7.2) to a density matching a #3 McFarland ($BASO_4$) opacity standard.

Enzyme Pretreatment: In those tests (ConA neg.) requiring enzymatic pretreatment of the yeast cells all enzymes were used in a final total concentration of 50 µg/ml. Yeast-enzyme mixtures were incubated for 1 hr at 37° C, with the exception of Proteinase K, which was incubated at 60° C. The enzymatically treated cells were then sedimented by centrifugation, and the pellets washed 2 times with PBS. After washing, the cells were suspended in PBS and adjusted to the standardized density (#3 McFarland Tube).

Performance of the Lectin-Agglutination Tests:

Standardized agglutination tests were performed by adding 1 drop of whole cell yeast antigen to a well in a Boerner slide, then adding 1 drop of lectin solution. Slides were placed on a rotary shaker for 10 min, and read macroscopically for signs of agglutination.

Lectins and/or Lectin-Like Substances:

All lectins were obtained from E-Y laboratories in either crude or purified form. The lectin-like agglutinins from Mangifera indica and Persea americana were extracted in our own laboratories. Extracts were freeze-dried for future use.

Table 10

Lysis of vegetative cells of *Bacillus species* by agents which dissipate protonmotive force[a].

Organism	CCCP[b]	Valinomycin[c]	Gramicidin[c]
B. subtilis 168	1.00	1.00	1.00
B. anthracis ATCC 11966	<0.01	<0.01	<0.01
B. megaterium	0.02	0.02	0.04
B. licheniformis	0.52	0.42	0.41
B. cereus T	<0.01	<0.01	<0.01
B. cereus ATCC 19637	<0.01	<0.01	<0.01
B. thuringiensis 4040	0.28	0.15	0.21
B. circulans 7049	0.58	0.46	0.74
B. amyloliquefaciens	0.43	ND	0.48
B. brevis	<0.01	0.16	<0.01
B. polymyxa	0.29	0.32	0.35
B. pumilus	0.15	<0.01	0.19

[a]Vegetative cells were washed in 50 mM phosphate buffer containing 0.5% (w/v) D-glucose and suspended to a density of 0.7-0.8 absorbance units (600 nm, 1cm) at room temperature. First order rate constants for loss of turbidity were calculated. Values shown above are relative to *B. subtilis* 168.

[b]CCCP, carbonylcyanide-m-chloro-phenylhydrazone (40μM final concentration).

[c]Valinomycin, 20μg/mL ; gramicidin , 2μg/ml.

ıble 9

Susceptibilities of Bacillus species to lysozyme and mutanolysin[a]

Organism	Lysozyme[b]	Mutanolysin[b]
B. subtilis 168	1.00	1.00
B. subtilis W23	0.95	1.16
B. megaterium KM	0.11	5.00
B. licheniformis	0.18	0.10
B. brevis	0.18	0.58
B. sphaericus	0.35	1.79
B. anthracis ATCC 14185	0.02	0.61
B. anthracis ATCC 11966	<0.01	0.73
B. thuringiensis ATCC 4040	<0.01	0.32
B. thuringiensis ATCC 4045	<0.01	0.54
B. cereus T	<0.01	0.17
B. cereus ATCC 6464	<0.01	0.52
B. cereus ATCC 9634	<0.01	0.14
B. cereus ATCC 19637	<0.01	0.37
B. circulans 7049	0.06	10.2
B. coagulans	0.32	0.74
B. polymyxa	0.04	8.10
B. amyloliquefaciens N	0.50	2.78
B. cereus var. mycoides	0.07	<0.01

[a]Heat-inactivated whole cells (100° C, 15 min) were suspended to an absorbance of 0.7-0.8 (1-cm, 500 nm) in 50mM sodium phosphate, 150 mM sodium chloride (pH 7.2). The cells were incubated with 50µg / ml egg-white lysozyme or 50µg/ml mutanolysin (final concentrations of the enzyme preparations). Decrease in turbidity was measured at room temperature (21-23°C) at 500 nm, 1-cm.

[b]Values shown are relative to the rate of clearing of B. subtilis 168. Rates were calculated as first order constants, derived from linear portions of log absorbance versus time (min).

For lysozyme the K for B. subtilis 168 was 1.59 hr. $^{-1}$. For mutanolysin, the K for B. subtilis 168 was 0.08 hr^{-1}.

Table 8

Composition of cell walls of *Bacillus* species[a]

Organism	Phosphorus	Hexose (μmol/mg)	Uronic Acid
B. anthracis 125 V770	<0.1	0.98	0.16
B. anthracis 119 V770	<0.1	0.85	0.15
B. anthracis 129 V770	<0.1	0.74	0.12
B. anthracis 112 Sterne	<0.1	0.61	0.14
B. anthracis 113 Sterne	<0.1	0.89	0.18
B. anthracis 120 Sterne	<0.1	0.48	0.11
B. anthracis V3 (heat cured)	<0.1	0.67	0.12
B. anthracis V1 (heat cured)	<0.1	0.84	0.15
B. anthracis Sterne (heat cured)	<0.1	0.90	0.16
B. anthracis V770 (heat cured)	<0.1	0.56	0.11
B. cereus E14579	<0.1	0.37	0.09
B. cereus 19637	<0.1	0.74	0.14
B. thuringiensis (USAMRIID)	<0.1	0.42	0.06
B. subtilis 168	1.19	0.90	0.15
B. subtilis W23	1.35	0.97	0.16

[1]Glycerol, ribitol - negative in all except *B. subtilis*. In *B. subtilis* 168, glycerol was 1 μmol/mg. In *B. subtilis* W23, ribitol was detected using TLC and periodate Schiff's reagent.

Hexose assay: anthrone reagent, D-glucose stdn

Uronic acid: carbazole reagent, D-glucuronic acid stdn;

Methyl pentose: cysteine H_2SO_4, L-rhammose stdn.; absent in all samples.

Sialic acid: thiobarbituric acid; crystalline sialic acid (Sigma); absent in all samples

Table 7

(cont'd.)

[a]Cells were grown for 3 days, with shaking, in penassay broth containing 10mM $CaCL_2$. The suspensions were then incubated with 0.1°/₀ (w/v) sodium azide in order to activate autolysins. The suspensions were then centrifuged and washed twice in distilled water and finally suspended in the same. The suspensions were then centrifuged (20,000 xg) in a solution of 55°/₀ (w/v) sucrose. The sediment was again suspended in water and re-centrifuged in 55°/₀ sucrose. After four sedimentations in sucrose, the spores were washed two times in PBS and finally resuspended in PBS containing 0.1°/₀ sodium azide. These suspensions, when checked by the Gram stain, revealed only occasional rod-shaped forms. The suspensions were then used in the lectin assays.

Lectins which did not agglutinate with any of the spores included LPA, lotus, UEA-I, PEA, BPA, PLA, DBA, STA, SJA, AND PHA-E. Conditions for the assays were the same as those described in Table 3. Only results from the rapid slide agglutination tests are reported in this progress. ±, indicates variable results showing possible differences in different batches of spores.

Table 7

Bacillus spores and Lectin Agglutination Tests[a]

Group I	RCA I	RCA II	SBA	APA	BSA I	BSA II	CON A	WGA	UEA II	MPA	RPA	HPA	SRA	HAA
B. anthracis 11966	+	+	+	+	+	-	-	-	-	-	-	-	-	-
B. anthracis 14185[a]	+	+	+	+	+	-	-	-	-	-	-	-	-	-
B. cereus T	-	-	-	-	-	+	-	+	-	-	-	+	-	+
B. cereus 6464	-	-	-	+	-	-	-	-	-	-	-	+	-	+
B. cereus 9634	-	-	-	-	-	-	-	-	-	-	-	+	-	+
B. cereus 23260	-	-	-	-	-	-	-	-	+	+	+	-	+	+
B. cereus E14579	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus 19637	-	-	-	-	-	-	+	-	-	-	-	+	-	+
B. cereus 246	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B. cereus/mycoides	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. thuringiensis 4040	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. subtilis 168	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B. subtilis W23	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. megaterium KM	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. licheniformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6

(cont'd.)

small clumps; 2+, most cells in fine clumps; 1+, mixture of finely dispersed aggregates and unclumped cells; 0, no evidence of aggregates.

Table 6

Reversal of autoagglutination of *Bacillus* species[a]

Organism	PBS	SDS	DNAase	Trypsin	Subtilisin	Succinic anhydride	100° C (30 min)	Autoclaved
B. anthracis 14185	4+	4+	4+	4+	4+	4+	1+	0
B. cereus 19637	4+	4	4+	4+	4+	4+	1+	1+
B. cereus 7064	4+	0	4+	4+	0	2+	1+	0
B. cereus 23260	1+	0	1+	1+	3+	0	0	0
B. cereus/mycoides	3+	2+	3+	3+	3+	2+	0	0
B. megaterium	4+	4+	1+	4+	2+	1+	1+	0
B. megaterium (exponential)	4+	3+	4+	3+	1+	1+	0	0
B. megaterium (stationary)	3+	1+	4+	3+	3+	1+	0	0

[a]Cells were recovered from 18 hr cultures on typticase blood agar base plates and suspended in 50 mM phosphate, 150 mM sodium chloride buffer (pH 7.2, PBS). Suspensions were washed once prior to digestion or treatments. The final concentration of SDS was 0.1 % (wt/vol). Trypsin and subtilisin concentrations were 250 µg/ml, whereas DNAase was 100 µg/ml. Enzyme treatments were for 4 hr at 37°C. Succinic anhydride (5 mg/ml in acetonitrile) was added to a final concentration of 200 µg/ml to the suspensions in PBS. Exponential and stationary phase cells of *B. megaterium* were obtained by growth in penassay broth with aeration at 37°C. Growth was monitored by light scattering. Arbitrary grading systems were established as: 4+, large macroscopic aggregates; 3+, large aggregates, some cells in

Table 5

Agglutination of Selected *Bacillus* species by Lectins [a].

	RCA-I	RCA-II	SBA	APA	BSA-II	BSA-I	ConA	WGA	PEA	RPA	BPA	STA	HPA	PNA	SRA	HAA	LPA
Group II																	
B. brevis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. lentus	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Group III																	
B. spaericus	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. globisporus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group IV																	
B. subtilis 168	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. subtilis W23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. amyloliquefaciens N	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
B. licheniformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. megaterium KM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B. pumilus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group V																	
B. circulans 7049[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. polymyxa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Group VI																	
B. coagulans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Table 3 describes conditions for reactions.

Table 4

Group I *Bacillus* non-reactive with lectins[a].

Bacillus	Lectin
B. cereus 6464	RCA-I, RCA-II, SBA, APA, BSA-I,
B. cereus 9634	BSA-II, conA, LPA, WGA, lotus,
B. cereus 13472	UEA-I, UEA-II, MPA, PEA, RPA, BPA,
	DBA, STA, SJA, PHA-E, HPA, PNA,
B. cereus var. mycoides (UM)	SRA, HAA
B. thuringiensis 4040	
B. thuringiensis 4041	
B. thuringiensis 4042-B	
B. thuringiensis 4045	
B. thuringiensis 4055	
B. thuringiensis 4060	

Table 3

Interaction Between Lectins and Group I *Bacillus* species[a].

ORGANISM	Lectin																
	RCA-I	RCA-II	SBA	APA	BSA-I	BSA-II	ConA	WGA	PEA	RPA	BPA	STA	HPA	PNA	SRA	HAA	LPA
B. anthracis 11966	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
B. anthracis 14185	+	+	+	+	+	-	-	-	-	-	-	-	-	-	±	-	-
B. cereus 4915	-	-	-		-	-	-	+	-	-	-	-	-	-	-	-	-
B. cereus 9620	-	-	-		+	-	+	+	-	-	-	-	-	-	-	-	-
B. cereus 7064	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus 11778	-	-	-	-	+	-	-	+	-	-	-	-	+	-	-	-	-
B. cereus E14578	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-
B. cereus 23260	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	-
B. cereus 246	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
B. cereus T	-	-	-	-	-	-	-	-	-	-	-	-	±	-	-	-	+
B. cereus 19637	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus var. mycoides	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
B. thuringiensis 4040	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Lectins (50 µL if 200 µg/mL) were mixed with vegetative cells (50 µL of 0.5 D 500 nm suspension in PBS) by gentle shaking at room temperature for 10 min. The mixtures were then visually examined for agglutination. +, indicates marked evidence of agglutinations; -, no agglutination - similar to controls without lectins.

Table 2

Groups of *Bacillus* based on the API Classification

Group I

- *B. anthracis*
- *B. cereus*
- *B. cereus* var. mycoides
- *B. thuringiensis*

Group II

- *B. brevis*
- *B. lentus*

Group III

- *B. sphaericus*
- *B. globisporus*

Group IV

- *B. subtilis*
- *B. amyloliquefaciens*
- *B. pumilus*
- *B. megaterium*
- *B. licheniformis*

Group V

- *B. circulans*
- *B. polymyxa*

Group VI

- *B. coagulans*

Table 1

Lectins used to Agglutinate *Bacillus* species

Lectin	Abbreviation	Specificity
Abrus precatorius	APA	D-Gal
Arachis hypogeae (peanut)	PNA	D-Gal-β-(1→3)-GalNAc
Bandeiraea simplicifolia	BSA-I	α-D-Gal
Bandeiraea simplicifolia	BSA-II	D-GlcNAc
Bauhinia purpurea	BPA	D-GalNAc
Canavalia ensiformis (jackbean)	ConA	α-D-Glc, α-D-Man
Dolichos biflorus	DBA	α-D-GalNAc, D-Gal
Glycine max (soybean)	SBA	α-D-GalNAc, D-Gal
Helix aspersa (snail	HAA	αor β-D-GalNAc
Helix pomatia (snail)	HPA	α-D-GalNAc
Limulus polyphemes (horseshoe crab)	LPA	Sialic acid
Lotus tetragonolobus	Lotus	α-L-Fucose
Maclura pomifera (osage orange)	MPA	α-D-Gal
Phaseolus limensis (lima bean)	LBA	α-D-GalNAc
Phaseolus vulgaris (kidney bean)	PHA	D-GalNAc
Pisum sativum (pea bean)	PEA	α-D-Glc, D-Man
Ricinus communis (castor bean)	RCA-I	β-D-Gal
Ricinus communis	RCA-II	βD-Gal, D-GalNAc
Robina pseudoacacia	RPA	unknown
Salvia horminum	SHA	unknown
Solanium tuberosum (potatoe)	STA	$(\beta\text{-D-GlcNAc})_2$
Sophora japonica (Japanese pagoda)	SJA	α-D-GalNAx
Triticum vulgaris (wheat)	WGA	α-D-GlcNAc
Ulex europaeus	UEA-I	α-L-Fucose
Ulex europaeus	UEA-II	$(\text{D-GlcNAc})_2$

TABLES & ILLUSTRATIONS

PL-5

Progress:

As seen in Table 14, 100% of Candida and Saccharomyces genera tested gave positive agglutination reactions with Con A, whereas Cryptococcus neoformans and Rhodotorula species were all negative. Although not shown in the table, Pisum sativum, which has the same carbohydrate binding specificity (α-D mannose) as Con A, gave similar results. Table 1 also shows that none of the species of Candida were agglutinated when tested with lectin-like extracts such as Persea or Mangifera, whereas all species of Rhodotorula gave a positive agglutination reaction. Cryptococcus neoformans, when tested with these lectin-like substances, gave variable results. The differential problem in separating Rhodotorula from Cryptococcus could not be resolved by using serial decrements of the extracts as shown in Table 15. This problem was obviated, however, by pretreatment of the yeast cells with Proteinase K. (Table 16). After enzyme treatment, all the Cryptococcus strains gave a negative reaction with the lectin-like extracts, whereas the species of Rhodotorula remained positive.

Based on these data, we propose that the scheme shown in Diagram 1, provides a rapid procedure for the identification of Cryptococcus neoformans. This lectin agglutination test can be performed in approximately 90 min. Conventional techniques presently used, require a series of subcultures and a minimum of overnight incubation in order to read the biochemical test results.

Future Studies:

In order to confirm the reliability of the proposed test, a larger series of strains should be tested, not only in our laboratory but in others as well.

A larger sampling is indicated in order to determine whether or not a significant number of atypical reacting strains may be encountered among any of the genera of yeasts tested.

We also propose to continue testing new lectins and/or plant agglutinins for our original objective of finding a specific agglutinin for Cryptococcus neoformans. If such a species specific agglutinin can be found, it would eliminate the need for using ConA, as well as the proteinase K enzyme treatment in the presently proposed test. By so doing, the time required to perform the test would be reduced from 90 min to only 15 min.

Table 11

Dinitrophenylation of *Bacillus* cell walls

Organism	GlcNAc[a] (unmodified)	GlcNAc[a] (dinitrophenylated)	Unmodified/ dinitrophenylated
B. subtilis 168	535	126	4.25
B. cereus 19637	948	133	7.13
B. anthracis 14185	1384	83	16.7

[a]Values are nanomoles per mg cell wall. Analyses were performed on the amino acid analyzer after 16 hr hydrolysis of the samples in 4N Hcl.

Table 12

Random screen of lectins and plant extracts for agglutinating activity with *Francisella* and *Brucella* species

Lectins	F. tularensis (Army)	Br. abortus (BBL)	Br. melitensis (BBL)	Br. suis (BBL)
WGA	+	-	-	-
RCA	+(weak)	±	-	-
ConA	-	+	±	-
BSA-I,II	-	+	+	±
SJA	-	-	-	-
SBA	-	-	-	-
DBA	-	±	-	-
BPA	-	-	-	-
RPA	-	+	+	±
MPA	-	-	-	-
APA	-	-	-	-
LBA	-	-	-	-
LAA	-	-	-	-
HPA	-	-	-	-
Extracts				
Holly	-	-	-	-
Canteloupe	+	+	+	-
Persimmon	-	-	-	-
Acorn	-	-	-	-
Persea	-	-	-	+
Mango	±	+	+	+
Root Nodule	+	-	+	-

*Antigen Source: 1) *F. tularensis* - U.S. Army
2) *Brucella* - BBL

Table 13

Variation in Agglutinability
using antigens for different sources

Lectins	F. tularensis 1(a)	F. tularensis 2(b)	F. tularensis 3(c)	Br. abortus 2	Br. abortus 3	Br. melitensis 2	Br. melitensis 3
WGA	+	-	+	-	-	-	-
RCA	+	+	+	-	+	±	±
ConA	-	-	+	+	-	±	±
BSA-I, II	-	-	±	+	+	+	-
DBA	-	-	-	±	-	-	-
RPA	-	-	-	+	±	+	+
Extracts							
Cantelope	+	-	-	-	-	-	-
Persimmon	-	-	-	-	-	-	-
Persea	-	-	-	-	±	+	-
Mango	±	-	+	+	+	±	+
Soybean	+	-	±	-	-	-	-

a - Antigen prep obtained from Army

b - Antigen prep obtained from BBL Co.

c - Antigen prep obtained from Difco Co.

Table 14

INTERACTION OF CLINICALLY IMPORTANT YEASTS
WITH LECTINS OF DIFFERING SUGAR SPECIFICITIES AND WITH LECTIN-LIKE EXTRACTS

ORGANISM	No. Strains	AGGLUTINATION REACTIONS									
		LECTINS (1mg/ml)						(LECTIN-LIKE (2.5 µ/ml)			
		ConA		WGA		PHA		Persea americana		Mangifera indica	
		pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.
Candida albicans	15	15	0	0	15	0	15	0	15	0	15
C. tropicalis	13	13	0	0	13	0	13	0	13	11	2
C. pseudotropicalis	4	4	0	0	4	1	3	0	4	0	4
C. krusei	6	6	0	0	6	0	6	0	6	0	6
C. parapsilosis	10	10	0	0	10	0	10	0	10	0	10
C. stellatoidea	4	4	0	0	4	0	4	0	4	0	4
C. guillermundii	4	4	0	0	4	0	4	0	4	0	4
C. glabrata	10	10	0	0	10	0	10	0	10	0	10
Cryptococcus neoformans	12	0	0	0	0	0	0	1*	11	1*	11
Rhodotorula spps	9	0	0	0	0	0	0	9	0	9	0
Saccharomyces	8	8	0	0	8	0	8	0	8	0	8

Table 15

AGGLUTINATION OF *CRYPTOCOCCUS* AND *RHODOTORULA* WITH DILUTIONS OF MANGIFERA AND PERSEA

Organism	Extract	2.5	2.0	1.5	1.0	0.75	0.5	0.25	0.105	0.05
C. neoformans (7)	Mangifera	-	-	-	-	-	-	-	-	-
C. neoformans (6)		-	-	-	-	-	-	-	-	-
C. neoformans (B54)		+	+	+	+	+	-	-	-	-
R. glutinis		4+	4+	3+	3+	3+	2+	+	-	-
R. rubra		3+	3+	2+	2+	+	-	-	-	-
R. pilmanae		3+	3+	3+	2+	2+	2+	+	+	-
C. neoformans (7)	Persea	-	-	-	-	-	-	-	-	-
C. neoformans (6)		-	-	-	-	-	-	-	-	-
C. neoformans (B54)		+	+	-	-	-	-	-	-	-
R. glutinis		4+	3+	2+	+	-	-	-	-	-
R. rubra		+	+	+	-	-	-	-	-	-
R. pilmanae		4+	4+	3+	2+	+	+	+	+	-

Table 16

EFFECTS OF ENZYME PRETREATMENT
ON THE AGGLUTINATION OF CRYPTOCOCCUS NEOFORMANS
AND RHODOTORULA SPECIES

Organism	Enzyme Treatment	AGGLUTINATION REACTIONS			
		Mangifera indica		Persea	
		No. Pos.	No. Neg.	No. Pos.	No. Neg.
Cryptococcus neoformans (12 strains)	Trypsin	1	11	1	11
	Subtilisin	1	11	0	12
	Pronase	1	11	1	11
	Proteinase K	0	12	0	12
Rodotorula spps (3 spps)	Trypsin	3	0	3	0
	Subtilisin	3	0	3	0
	Pronase	3	0	3	0
	Proteinase K	3	0	3	0

*Final total con. of enzymes
1 hr at 60° C Prot. K All other[5] 37° for 1 hr cells washed 2 times

Table 17

Assay for presence of N. meningitidis in spinal fluids of humans[a].

No. tested[b]	No. postive[c]
114	6

[a]Spinal fluid (100μL) was centrifuged for 4 min in an Eppendorf centrifuge. Supernatants were decanted and 100 μL Tris-HCl (110 mM, pH 7.3) added to the tubes. The tubes were vortexed and 100 μL (containing 100 μg) substrate added (γ-glutamyl-4-methoxy-β-naphthylamide in Tris-HCl). Following an incubation of 1 hr at 37° 100 μL of coupling agent added (fast garnet dye forms an azo derivative with the liberated amine). A pink color denotes a positive reaction.

[b]All specimens were obtained from Dr. J. Snyder, Norton's-Children's Hospital, Louisville.

[c]The positive samples were shown to contain N. meningitidis by culturing techniques or counterimmuno-electrophoresis showed the presence of N. meningitidis antigens. Thus, to date, our assay is 100% accurate.

Table 18

Gamma-glutamyl aminopeptidase activity in selected bacteria and fungi[a].

Organism	Gamma-glutamyl aminopeptidase
Neisseria meningitidis (35 strains)	+
Neisseria gonorrhoeae (14 strains)	-
Streptococcus pyogenes	-
Streptococcus pneumoniae	-
Streptococcus faecalis	-
Staphylococcus aureus Wood 46	-
Staphylococcus epidermidis	-
Listeria monocytogenes	-
Escherichia coli	-
Hemophilus influenzae	-
Bacillus anthracis	-
Bacillus circulans	-
Bacillus coagulans	-
Bacillus cereus T	-
Candida albicans	-
Cryptococcus neoformans	-
Legionella pneumophila	+

[a]Substrate was γ-glutamyl-4-methoxy-β-naphthalamide.

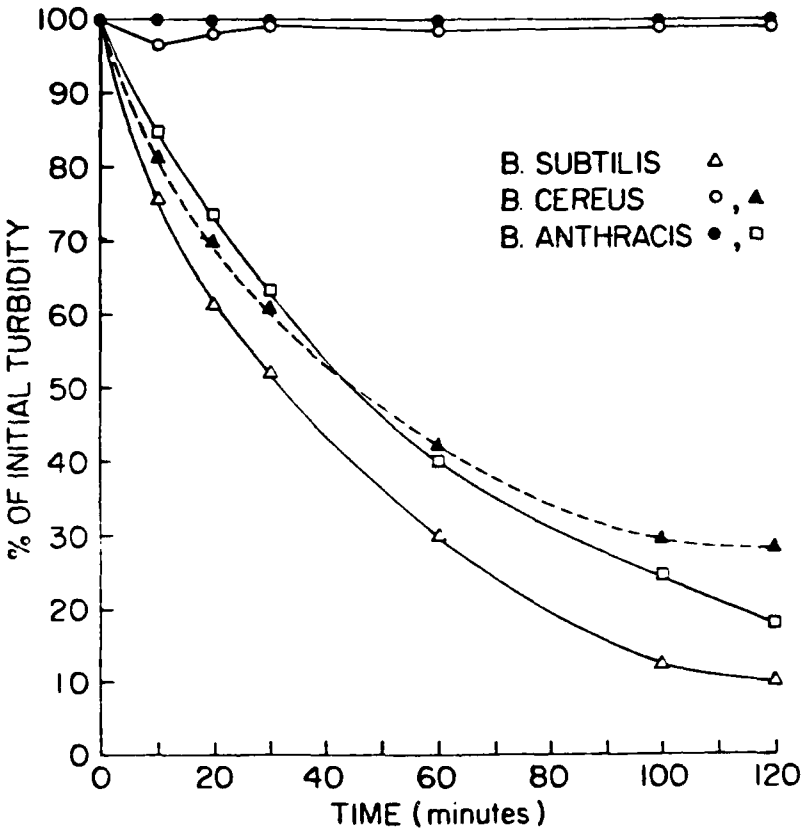
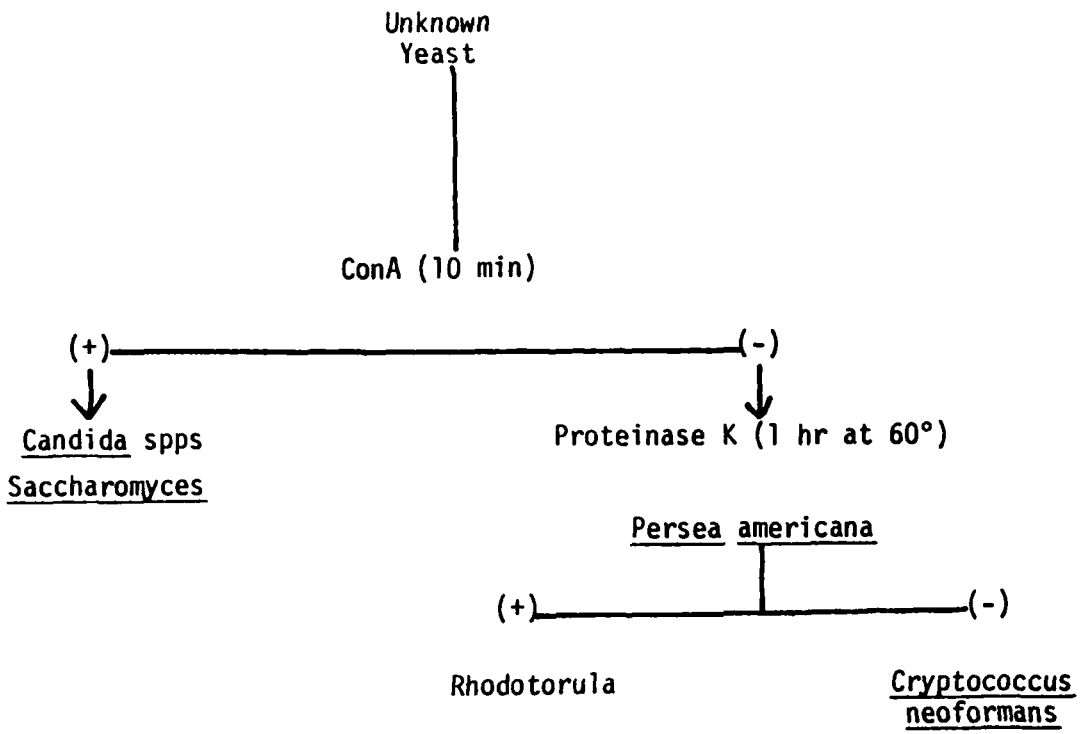


Fig. 1

Effect of acetylation of cell walls on susceptibilities to lysozyme. Walls were acetylated with acetic anhydride, incubated with 1.0 M NH_2OH to remove O-acetyl groups and washed extensively in phosphate buffer (pH 7.2). Lysozyme (final concentration of 50µg/mL) was added and the loss of turbidity determined by a spectraphatometer. Acetylated samples were ▲ and □. Strains were B. subtilis 168, B. cereus 19637 and B. anthracis 14185.

DIAGRAM 1


Unknown
Yeast
ConA (10 min)
(+)
(-)
Candida spps
Saccharomyces
Proteinase K (1 hr at 60°)
Persea americana
(+)
(-)
Rhodotorula
Cryptococcus
neoformans

END

FILMED

5-85

DTIC